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Preliminary Amendment

In the Specification:

Please replace the paragraph starting on page 1, line 3, with the following:

This application is a divisional application of United States Application No. 09/077,955 filed September 10, 1998, which claims priority of United States Application Serial No. 08/644,271 filed May 10, 1996 and of United States Provisional Application No. 60/008,657 filed December 15, 1995, each of which is incorporated by reference herein.

Please replace the paragraph starting on page 11, line 1, with the following:

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

The invention also provides for the above-described nucleic acid molecule which additionally contains a nucleotide sequence so that the encoded polypeptide contains the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14A-14C (SEQ ID NO: 34).

Please replace the paragraph starting on page 12, line 21, with the following:

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding human muscle specific kinase (MuSK) receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the human MuSK receptor as set forth in Figure 4A-4D (SEQ ID NO: 32);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes a human MuSK receptor; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a human MuSK receptor.

Please replace the paragraph starting on page 13, line 7, with the following:

FIGURE 1A-1D -Nucleotide (SEQ ID NO: 2) and deduced amino acid (single letter code) sequences (SEQ ID NO: 1) of rat musk. The nucleotide sequence encoding mature MuSK begins around nucleotide 192.

Please replace the paragraph starting on page 13, line 28, with the following:

FIGURE 4A - 4D -Nucleotide (SEQ ID NO: 32) and deduced amino acid (single letter code) sequences (SEQ ID NO: 33) of human MuSK receptor.

Please replace the paragraph starting on page 18, line 4, with the following:

FIGURE 14A-14C - Amino acid (single letter code) sequence (SEQ ID NO: 34) of rat agrin indicating Y and Z sites of amino acid inserts found in splice variants.

Please replace the paragraph starting on page 18, line 7, with the following:

FIGURE 15A-15B - Nucleotide (SEQ ID NO: 35) and amino acid (single letter code) sequences (SEQ ID NO: 36) of human agrin expression construct including the signal

peptide and flg tag (FLAG tag). The start of the coding region for the active C-terminal fragment (portion) of human agrin 4-8 is indicated. Also indicated are the position Y and position Z insert sites at which the 4 and 8 amino acid inserts are located. Throughout this application, references to human agrin 4,8; c-agrin 4,8; or human c-agrin 4,8 indicate the active C-terminal fragment (portion) of human agrin 4-8 as set forth in the Figure.

Please replace the paragraph starting on page 18, line 30, through page 19, line 2, with the following:

The present invention provides for a novel tyrosine kinase molecule that is related to the trk family of tyrosine kinases. The sequence of the protein is set forth in Figure 1A-1D as SEQ. ID NO: 1. The coding region of the mature protein is believed to begin on or around the serine-glycine-threonine on or around position 20 of the coded region.

Please replace the paragraph starting on page 19, line 19, with the following:

The gene encoding rat MuSK has been cloned and the DNA sequence determined (Figure 1A-1D; SEQ ID NO: 2). The extracellular domain of the mature protein is believed to be encoded by the nucleotide sequence beginning on or around position 192 and ending on or around position 1610. The transmembrane portion of the protein is believed to be encoded by the nucleotide sequence beginning on or around position 1611 and ending on or around position 1697. The intracellular domain is believed to be encoded by the nucleotide sequence beginning on or around position 1698 and ending on or around position 2738. A cDNA clone encoding Dmk (MuSK) was deposited with the American Type Culture Collection on July 13, 1993 and accorded an accession number of ATCC No. 75498.

Please replace the paragraph starting on page 20, line 22 through page 21, line 3, with the following:

The present invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding human muscle specific kinase (MuSK) receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the human MuSK receptor as set forth in Figure 4A-4D (SEQ ID NO: 32);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes a human MuSK receptor; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a human MuSK receptor.

Please replace the paragraph starting on page 24, line 16, through page 25, line 4, with the following:

The present invention further provides for substantially purified protein molecules comprising the amino acid sequence substantially as set forth in Figure 1A-1D for MuSK (SEQ ID NO: 1) or functionally equivalent molecules. Functionally equivalent molecules include derivatives in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are proteins or fragments (portions) or

derivatives thereof which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Please replace the paragraph starting on page 36, line 1, with the following:

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

The invention also provides for the above-described nucleic acid molecule which additionally contains a nucleotide sequence so that the encoded polypeptide contains the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14A-14C (SEQ ID NO: 34).

Please replace the paragraph starting on page 36, line 19, with the following:

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin as set forth in Figure 15A-15B (SEQ ID NO: 35);

(b) a nucleotide sequence that hybridizes under stringent conditions to the

nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

Please replace the paragraph starting on page 37, line 1, with the following:

The invention further provides for an isolated nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence as set forth in Figure 15A-15B (SEQ ID NO: 35);

(b) the nucleotide sequence encoding amino acids 24 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(c) the nucleotide sequence encoding amino acids 60 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(d) the nucleotide sequence encoding amino acids 76 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(e) the nucleotide sequence encoding amino acids 126 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(f) the nucleotide sequence encoding amino acids 178 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(g) the nucleotide sequence encoding amino acids 222 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(h) the nucleotide sequence encoding amino acids 260 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(i) the nucleotide sequence encoding amino acids 300 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(j) a nucleotide sequence that hybridizes under stringent conditions to any of the nucleotide sequences of (a) through (i) and which encodes the active portion of human agrin; and

(k) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from any of the nucleotide sequences of (a) through (j) and which encodes the active portion of human agrin.

Please replace the paragraph starting on page 37, line 29, through page 38, line 5, with the following:

A further embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 0-8 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set forth in Figure 15A-15B (SEQ ID NO: 35) with the exception that there is no insert at position Y. Another embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 4-0 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set forth in Figure 15A-15B (SEQ ID NO: 35) with the exception that there is no insert at position Z.

Please replace the paragraph starting on page 38, line 21, with the following:

Referring to Figure 15A-15B (SEQ ID NO: 35), starting at the N-terminal end (amino acid 24 - KSPC) these truncated forms of human agrin preferably have deletions of up to 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350 or 400 amino acids. Particularly preferred truncated forms are described herein as delta 3 through delta 9.

Please replace the paragraph starting on page 50, line 22, through page 51, line 2, with the following:

One of the cloned fragment sequences contained a segment of a novel tyrosine kinase domain, which was designated as MuSK. The sequence of the PCR-derived fragment corresponding to MuSK was used to generate PCR primers to obtain longer MuSK specific fragments by the RACE procedure. These longer MuSK probes were used as a hybridization probe to obtain full length MuSK cDNA clones

from a rat denervated skeletal muscle cDNA library. DNA was sequenced by using the ABI 373A DNA sequencer and Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of MuSK (Figure 1A-1D [SEQ ID NO:1]) has a high degree of homology to members of the trk family of proteins. It was also found to be similar to the Jennings, et al. Torpedo RTK found in muscle.

Please replace the paragraph starting on page 51, line 4, with the following:

Oligonucleotide primers corresponding to conserved regions of known tyrosine kinase molecules were used to amplify and clone DNA sequences encoding novel orphan tyrosine kinase receptor molecules. The amino acid sequences of representatives from branches of the tyrosine kinase family and regions of homology within the catalytic domain of these proteins were used to design degenerate oligonucleotide primers. These primers were then used to prime PCR reactions using as template a rat denervated muscle cDNA library. Resulting amplified DNA fragments were then cloned into Bluescript II SK(+) plasmid, sequenced, and the DNA sequences compared with those of known tyrosine kinases. The sequence of a PCR fragment encoding a novel tyrosine kinase named MuSK was used to obtain more adjoining DNA sequence. A DNA fragment containing MuSK sequences was used as a probe to obtain a cDNA clone from a denervated skeletal muscle library. This clone encodes a novel tyrosine kinase receptor with a high degree of homology to members of the trk family of proteins. It was also found to be homologous to the Jennings, et al. Torpedo RTK. Figure 1A-1D presents the nucleotide sequence (SEQ ID NO: 2) of the musk clone.

Please replace the paragraph starting on page 57, line 14, with the following:

This process was complemented by obtaining human genomic clones of MuSK and using the coding sequence of the genomic MuSK to design oligonucleotide primers used to amplify the biopsy cDNA. Stretches of the human MuSK cDNA sequence

which were difficult to sequence, absent or presenting some ambiguity were confirmed, corrected or added from the human genomic MuSK sequence. MuSK cDNA variants produced by alternative splicing of MuSK transcripts may be obtained by using this sequence to obtain MuSK cDNA from human sources. The deduced amino acid sequence of the human MuSK receptor and the nucleotide sequence encoding it is set forth in Figure 4A-4D (SEQ ID NO: 32). One of skill in the art will readily recognize that this sequence may be used to clone full length, naturally occurring cDNA sequences encoding the human MuSK receptor, which may vary slightly from the sequence set forth in Figure 4A-4D (SEQ ID NO: 32)

Please replace the paragraph starting on page 64, line 9, through page 65, line 2, with the following:.

The ability of various agrins and growth factors to induce MuSK or ErbB3 tyrosine phosphorylation, for the indicated times and at the indicated concentrations, was evaluated in primary rat myoblasts and in either untransfected C2C12 myoblasts, or in C2C12 myoblasts stably transfected with a chick MuSK-expressing plasmid. The cells were challenged at confluence in an undifferentiated state, or approximately 4-5 days after being induced to differentiate into myotubes in serum-poor media. After challenge, the cells were lysed, the extracts subjected to immunoprecipitation with receptor-specific antibodies, and then immunoblotted with either receptor-specific or phosphotyrosine-specific antibodies, using methods previously described (Stitt, T., et al., 1995, Cell 80: 661-670). Polyclonal antibodies for MuSK were generated as follows: for rat MuSK, rabbits were immunized with a peptide corresponding to the carboxy-terminal 20 amino acids of the rat MuSK protein (Valenzuela, D., et al., 1995, Neuron 15: 573-584; the nomenclature for this antibody is: 41101K); for chick MuSK, rabbits were immunized with a peptide corresponding to the first 19 amino acids of the chick MuSK cytoplasmic domain (Peptide: TLPSELLLDRLHPNPMYQ (SEQ ID NO: 16); the nomenclature for this antibody is 52307K). The specificity of the antibodies was determined on Cos-cell expressed MuSK proteins, by both immune-precipitation and Western, comparing untransfected Cos cell lysates to

lysates from rat and chicken-MuSK transfected Cos cells. 41101K immune precipitates and Westerns rodent MuSK, but does not recognize chicken MuSK. 52307 immune precipitates and Westerns chicken MuSK. Antibodies to ErbB3 were obtained from Santa Cruz Biotechnology, Inc.

Please replace the paragraph starting on page 75, line 5, with the following

Primer pair 18:
h-agrin 18-5' : 5'-GACGACCTCTTCCGGAATTC-3' (SEQ ID NO: 17)
h-agrin 18-3' : 5'-GTGCACATCCACAATGGC-3' (SEQ ID NO: 18)

Please replace the paragraph starting on page 75, line 9, with the following:

Primer pair 35:
h-agrin 35-5' : 5'-GAGCAGAGGGAAGGTTCCCTG-3' (SEQ ID NO: 19)
h-agrin 35-3' : 5'-TCATTGTCCCAGCTGCGTGG-3' (SEQ ID NO: 20)

Please replace the paragraph starting on page 76, line 18, with the following:

As a result of this screen, one clone (pBL-hAgrin1) was obtained which contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned nucleotide sequence corresponds approximately to amino acid 424 of rat agrin (See Figure 14A-14C). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin1 contains a 4 amino acid insert starting at the position which corresponds to position 1643 of Figure 14A-14C, a point which was previously described for the rat as position "Y" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the 4 amino acid insert both in clone pBL-hAgrin1 and in the rat is KSRK.

Please replace the paragraph starting on page 76, line 29, through page 77, line 14, with the following:

A second clone was obtained from this screen. This second clone (pBL-hAgrin23) also contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned nucleotide sequence corresponds approximately to amino acid 1552 of the rat agrin (See Figure 14A-14C [SEQ ID NO: 34]). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin23 contains an 8 amino acid insert starting at a position which corresponds to position 1780 of Figure 14A-14C (SEQ ID NO: 34), a point which was previously described for the rat as position "Z" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the eight amino acid insert both in clone pBL-hAgrin23 and in the rat is ELANEIPV. As previously discussed, it has been reported that the 8 amino acid insert plays an important role in regulating the AChR clustering activity of different agrin forms. Therefore, by inserting a nucleotide sequence encoding the eight amino acid sequence ELANEIPV into clone pBL-hAgrin1 at the position corresponding to position Z of rat agrin, a human 4-8 agrin clone may be obtained. The addition of the 8 amino acid insert at position Z should confer a high level of biological activity to the human 4-8 clone.

Please replace the paragraph starting on page 77, line 30, through page 78, line 14, with the following:

A human agrin Sfi I - Aat II fragment containing the 4 amino acid insert at the position corresponding to the Y-site described for rat agrin (see Figure 14A-14C [SEQ ID NO: 34]) was excised from clone pBL h agrin-1. A human agrin Aat II - Not I fragment containing the 8 amino acid insert at the position corresponding to the Z-site described for rat agrin (see Figure 14A-14C [SEQ ID NO: 34]) was excised from clone pBL h agrin-23. A Xho I - Sfi I fragment was then generated via PCR that contained a preprotrypsin signal peptide, the 8 amino acid flg peptide (from the flag tagging system, IBI/Kodak, Rochester, NY) and the human agrin sequence corresponding to the sequence of amino acids from position 1480 to the Sfi I site located at amino acids 1563-1566 of rat agrin (see Figure 14A-14C [SEQ ID NO: 34]).

The three fragments were then ligated into a Xho I - Not I digested pMT21 expression vector to form the human agrin 4-8 expression vector pMT21-agrin 4-8. The sequence of human agrin 4-8 that was encoded in the expression vector is shown in Figure 15A-15B (SEQ ID NO: 35). Expression vectors for the human clones corresponding to splice variants containing (Y-Z) inserts of (0-8) and (4-0) were also constructed.

Please replace the paragraph starting on page 78, line 18, with the following:

The gene for human agrin 4-8 was PCR amplified from pMT21-agrin 4-8 with the primer pair AG5' (5'-GAGAGAGGTTTAAACATGAGCCCCTGCCAGCCCAACCCCTG-3' [SEQ ID NO: 21]) and AG3' (5'-CTCTGCGGCCGCTTATCATGGGGTGGGGCAGGGCCGCAG-3' [SEQ ID NO: 22]). The PCR product was digested with the restriction enzymes Pme I and Not I and cloned into the Pme I and Not I sites of the vector pRG501, a pMB1 replicon that confers kanamycin resistance and is designed to express cloned genes from the phage T7 promoter. One isolate was characterized and named pRG531. The 1315 base pair Nco I - Nae I fragment internal to agrin in pRG531 was then replaced with the corresponding fragment from pMT21-agrin 4-8. The resulting plasmid, pRG451, was transformed into the expression strain RFJ209 [IN(rrnD-rrn/E)1 $lacI^{Q}$ lacZpL8 fhuAD322-405 $rpoS_{(MC4100)}$ ara::(lacUV5-T7 gene 1)8]. Cultures of RFJ209 / pRG541 induced with IPTG express human agrin to about 5% of total cellular protein and fractionates with soluble protein upon cell disruption. The crude soluble protein fraction containing human agrin 4-8, as well as human agrin 4-8 purified by Q-Sepharose chromatography was determined to be active in phosphorylation of MuSK receptor.

Please replace the paragraph starting on page 79, line 8, with the following:

The 50kD active fragment (portion) of human agrin 4-8 was cloned by PCR using a primer containing a portion of the S. cerevisiae α mating factor pre-pro secretion signal and the 5' end of the region encoding the 50kD agrin fragment (GTATCTCTCGAGAAAAGAGAGGCTGAAGCT AGCCCCTGCCAGCCCAACC [SEQ ID NO: 23]), and a primer containing sequences from the region 3' of the agrin coding region and a NotI site (AATAGTGCGGCCGCCAACACTCAGGCAAGAAAATCATATC [SEQ ID NO: 24]). After PCR the fragment was digested with XhoI, which recognizes sequences in the 5' primer, and NotI, and was cloned into pPIC9 (Invitrogen) digested with XhoI and NotI. The resulting clone was digested with NotI and partially digested with NcoI to remove most of the PCRed agrin sequences. This region was replaced by a NotI-NcoI fragment of agrin from pRG541. PCRed regions were sequenced and shown to be wild-type. This clone, pRG543 was digested with SalI and transformed into Pichia pastoris by electroporation. Transformants were selected for a His+ Mut+ phenotype. Induction of the AOX1 promoter driving the expression of hAgrin was done by growing the cells in buffered glycerol-complex medium containing 0.5% glycerol, pH=6.0, for 24 hrs until the glycerol was exhausted, at which point methanol was added to a final concentration of 0.5%. The culture was centrifuged and the supernatant was dialyzed against PBS. The concentration of hAgrin was approximately 10ug/ml and was determined to be active in phosphorylation of MuSK receptor.

Please replace the paragraph starting on page 83, line 1 with the following:

As set forth in Figure 15A-15B (SEQ ID NO: 36), the amino acid sequence of the 50 kD active portion of human agrin 4,8 is 492 amino acids long. A preprotrypsin signal sequence (Stevenson et al., 1986. Nucleic Acids Res. 21: 8307-8330) precedes a FLAG tag sequence (Hopp et al. 1988. Bio/Technology 6: 1204-1210); together, they constitute the first 23 amino acids. Thus the agrin 4,8 sequence begins with amino acid 24. Truncated molecules were created, each of which contained the signal sequence and FLAG tag (23 amino acids) followed by the agrin 4,8 sequence to